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Islet autoantibody profiles associated with higher diabetes risk in Lithuanian compared with English schoolchildren

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Summary

During a 15-year period, the incidence of type 1 diabetes has doubled in Lithuania, while increasing by a third in England; however, England still has a higher incidence. Analysis of sera collected from non-diabetic schoolchildren from Lithuania and England more than 20 years ago showed a similar number of multiple autoantibody-positive schoolchildren between the populations, but a higher prevalence of islet antigen-2 autoantibodies (IA-2A) in English schoolchildren. We aimed to use recently developed, more specific islet autoantibody tests to characterize differences in humoral autoimmunity between these two general population cohorts in greater detail. Samples from 88 Lithuanian and 133 English schoolchildren previously found islet autoantibody-positive were selected for measurement of additional islet autoantibodies by radioimmunoassay. Samples were tested for autoantibodies to zinc transporter 8 (ZnT8A), GAD (96–585), the protein tyrosine phosphatase region of islet antigen-2 (PTPA) and the related IA-2βA, while autoantibodies to IA-2A were reassayed using the current harmonized method. IA-2-related autoantibodies PTPA (0.13 versus 0.45%, $P = 0.027$) and IA-2βA (0 versus 0.35%, $P < 0.001$), but not IA-2A measured using the harmonized method, were less common in Lithuanian compared to English schoolchildren. Lithuanian schoolchildren who were islet autoantibody-positive were positive for fewer biochemical autoantibodies compared with English schoolchildren ($P = 0.043$). Background rates of islet autoimmunity in childhood differ subtly between countries, which have different incidences of type 1 diabetes. The optimal screening strategy (age and combination of markers) for detection of islet autoimmunity may vary between countries, dependent upon the pattern of autoantibodies found in the general population.

Keywords: autoantibodies, immunophenotype, rising incidence, schoolchildren

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Introduction

Environmental factors are often cited as an explanation for differences in incidence of type 1 diabetes; within Europe the incidence ranged approximately 10-fold from 5.8 of 100 000 (Macedonia) to 56 of 100 000 (Finland) between 2000 and 2005 [1]. We found previously that the prevalence of autoantibodies to islet antigen-2 (IA-2A) was 10-fold lower (0.2 versus 2.4%) in 3053 schoolchildren from Lithuania, with a diabetes incidence

of 7.1 cases per 100 000/year, compared with 2860 schoolchildren of a similar age from the United Kingdom, which at that time had an incidence of 17 cases per 100 000/year [2]. The frequency of autoantibodies to insulin (IAA), glutamate decarboxylase (GADA) and multiple autoantibodies [mAb, which included islet cell antibodies (ICA)] was similar in the two groups. Although measurement of biochemical autoantibodies (where the antigen is known) is now common, ICA are still included as a secondary measure within TrialNet, where their

inclusion aided disease prediction [3]. A later study comparing islet autoantibody prevalence in schoolchildren with similar ethnic origins (Finland and Russian Karelia), but different incidences of type 1 diabetes, also found an increased IA-2A prevalence in schoolchildren from the population with a higher incidence [4]. Since our original paper, autoantibodies to zinc transporter 8 (ZnT8A) have been discovered and shown to be useful for disease prediction. Both ZnT8A and IA-2 β A, a homologue of IA-2A, are associated with rapid progression to disease [5]. An international harmonized protocol for measurement of IA-2A has also been established [6], while measurement of autoantibodies using a truncated radiolabel [GAD(96–585)] improves specificity compared with using full-length antigen [7].

We previously investigated autoantibody prevalence at diagnosis in patients from the same geographical region as the English schoolchildren during a 17-year time-span, when type 1 diabetes incidence rose and prevalence of ZnT8A and IA-2A increased [8]. The prevalence of IA-2 β A and autoantibodies recognizing the protein tyrosine phosphatase region of IA-2 (PTPA) also increased during this time-span. Given our previous data and the association between diabetes incidence and prevalence of ZnT8A and IA-2A, we hypothesized that autoantibodies associated with a higher risk of diabetes [ZnT8A, GADA (96–585) and IA-2A/IA-2 β A epitopes] would also differ in the Lithuanian and English schoolchild populations.

Methods

The Lithuanian and English schoolchild samples have been previously described [2]. Serum samples from 3053 Lithuanian schoolchildren were available for study; the median age was 11.7 years (age range = 5.5–15.0 years), with 1449 (47%) boys. Samples from 2860 English schoolchildren were also available; the median age was 11.4 years (age range = 9.0–13.8 years), with 1488 (52%) boys. Samples have been stored at -20°C since sample collection between 1994 and 1998. The two sample collections have been approved by local ethics committees and the study was performed according to the principles of the Declaration of Helsinki.

Autoantibodies for GAD (96–585), IA-2, IA-2 β and PTP were measured as previously described [6–8]. Islet autoantibody units were derived from standard curves of serially diluted positive sera. The threshold for positivity was set at 1.8 units; the 97.5th percentile of 523 of the non-diabetic English schoolchildren for autoantibodies was measured using ZnT8 (325R) or ZnT8 (325W). The positivity threshold for GAD (96A) was set at 12.8 units, the 97.5th percentile of 222 English

schoolchildren. The threshold for IA-2A measured using the harmonized method was set at 1.4 DK units/ml, the 99.2nd percentile of 500 non-diabetic adult controls [6]. For PTPA and IA-2 β A the thresholds were set at 3 standard deviations (s.d.) of 270 of the English schoolchildren; 0.57 and 0.93 units, respectively. Samples were measured in duplicate; where the error was $\geq 30\%$ the results were inspected and samples with results close to the threshold for positivity were repeated. Samples were also repeated where negative, low, medium or high positive controls were out of range.

Statistical analysis

The percentage of people positive for a given autoantibody was compared between Lithuanian and English schoolchildren using Fisher's exact test. The number of islet autoantibodies was compared using the χ^2 test. Mann–Whitney *U*-testing was used to compare autoantibody titres within individuals positive for each autoantibody. Statistical analysis was carried out using Prism version 6 software (GraphPad Software, Inc., La Jolla CA, USA). A *P*-value < 0.05 was considered significant.

Results

ZnT8A and GADA

Data for GADA, IA-2A, IAA and ICA tested historically were already available; originally, ICA and IAA were only tested in GADA- and/or IA-2A-positive individuals ($n = 221$), with 27 (12.2%)- and 19 (8.6%)-positive, respectively. Samples previously positive for GADA and/or IA-2A from schoolchildren (88 Lithuanian and 133 English schoolchildren, Fig. 1) were selected for measurement of ZnT8A using published methods [8]. Of these, samples from one Lithuanian and one English child (who were not mAb-positive) were not of sufficient volume for ZnT8A measurement. Schoolchildren negative for GADA and IA-2A (2965 Lithuanian and 2727 English schoolchildren) were considered ZnT8A negative, because in an unpublished screen of 1400 English 7-year-olds negative for IA-2A and GADA only four were ZnT8A-positive, and other studies including The Environmental Determinants of Diabetes in the Young (TEDDY) also use this protocol [9].

Overall, four (0.13%) Lithuanian and six (0.14%) English schoolchildren tested positive for ZnT8A [*P* = not significant (n.s.), Supporting information, Fig. S1a]. In previous GADA-positive schoolchildren, GADA were remeasured using GAD (96–585), 47 of 80 (58%) Lithuanian and 36 of 70 (51%) English schoolchildren were positive for autoantibodies recognizing GAD (96–585) (*P* = n.s., Supporting information, Fig. S1b). Truncated GADA screening identified almost all mAb positives, but only half of

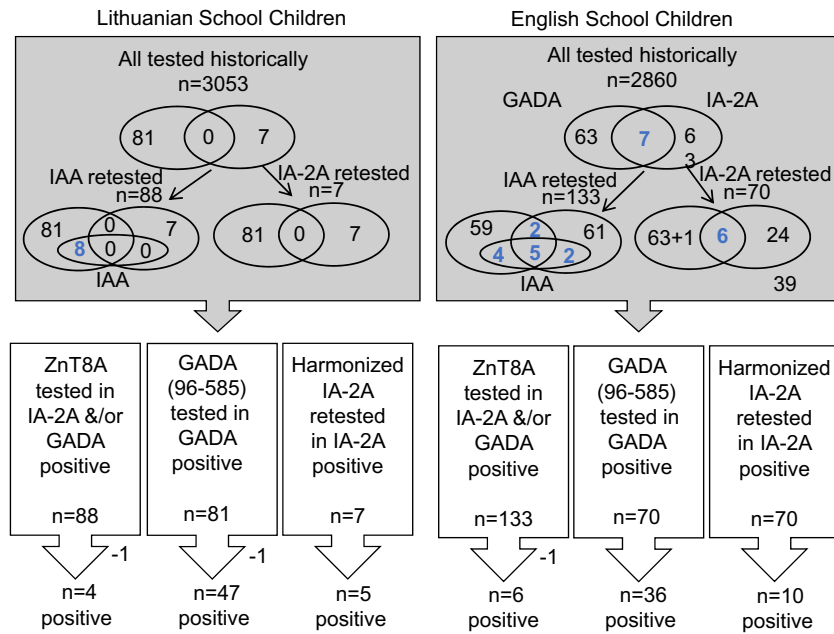


Fig. 1. Testing strategy. Historic islet autoantibody testing [2] is summarized in the grey boxes, including retesting of islet antigen-2 autoantibodies (IA-2A) and measurement of autoantibodies to insulin (IAA) in samples positive for IA-2A and/or autoantibodies to glutamic acid decarboxylase (GADA). Previous results were used to select samples for current islet autoantibody testing. The autoantibody testing strategy in the current study is indicated in the white boxes. Autoantibodies to zinc transporter 8 (ZnT8A) were tested in samples previously positive for IA-2A and/or GADA. GADA (96-585) were tested in samples previously GADA-positive. IA-2A-related autoantibodies were tested in samples previously positive for IA-2A. Samples that could not be tested are indicated by minus symbols next to the arrows. Multiple autoantibody-positive individuals are indicated in bold blue text.

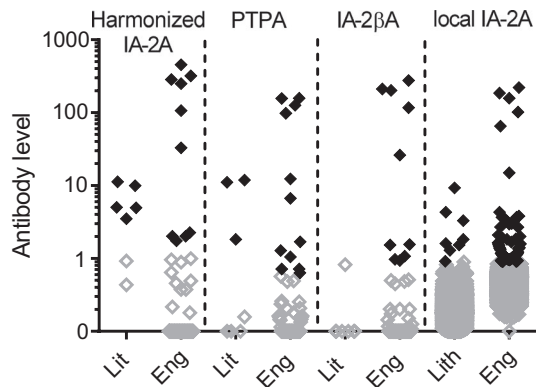


Fig. 2. Titres of islet antigen-2 autoantibodies (IA-2A) in Lithuanian (Lit, $n = 7$ or 3053) and English (Eng, $n = 70$ or 2860) schoolchildren. With IA-2A measured using the harmonized method (threshold 1.4 DK units/ml), protein tyrosine phosphatase region of IA-2 (PTPA) (threshold 0.57 units), IA-2 β A (threshold 0.93 units) and local IA-2A (threshold 0.94 units). Black diamonds = positive individuals, grey diamonds = negative individuals. Differences in titre were not statistically significant within individuals who were autoantibody-positive.

the school children were previously GADA-positive, suggesting that it identifies schoolchildren at higher risk of progression to diagnosis of diabetes.

IA-2 related autoantibodies

In seven Lithuanian and 70 English previously IA-2A-positive schoolchildren, IA-2A, IA-2PTPA and IA-2 β A were measured using the harmonized method [6,8]. When IA-2A were remeasured, five (0.16%) Lithuanian and 10 (0.35%) English schoolchildren were positive ($P = \text{n.s.}$, Supporting information, Fig. S1c). In contrast, however, PTPA and IA-2 β A were less common in Lithuanian schoolchildren (0.10 and 0% of 3053, respectively) compared with English schoolchildren (0.42 and 0.35% of 2860, with $P = 0.018$ and $P < 0.001$, respectively, Supporting information, Fig. S1d). These results were mirrored, although not statistically different for IA-2A titres (Fig. 2). Several English schoolchildren had titres of IA-2A, PTPA and IA-2 β A more than 20-fold higher than any of the Lithuanian schoolchildren.

Number of autoantibodies

Overall, 24 schoolchildren (10 Lithuanian and 14 English) were mAb-positive by combinations of GADA/IA-2A/IAA/ZnT8A testing (Supporting information, Fig. S1a). This number increased to 41 (24 Lithuanian and 17 English) if ICA were considered an independent autoantibody (Supporting information, Fig. S2). Interestingly,

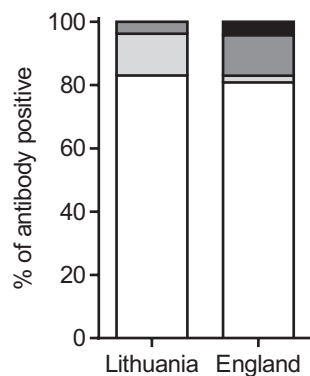


Fig. 3. Number of autoantibodies as a percentage of the 53 Lithuanian and 47 English schoolchildren found autoantibody-positive using updated assays. Based on autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA) (96–585), harmonized islet antigen-2 autoantibodies (IA-2A) (and/or IA-2 β A) and zinc transporter 8 (ZnT8A) (ESM Fig. S1c). Black = 4, dark grey = 3, light grey = 2, white = 1 autoantibody. A smaller proportion of Lithuanian schoolchildren had three to four autoantibodies compared with English schoolchildren ($P = 0.043$).

ICA was more prevalent in the Lithuanian schoolchildren with autoantibodies recognizing GAD (96–585) and full-length GAD [12 of 50 (24%)], compared with those with autoantibodies recognizing full-length GAD alone [two of 32 (6.3%, $P = 0.037$)]. The number of Lithuanian schoolchildren considered mAAb-positive doubled when ICA was considered. When newer, more specific methods were used, only 18 schoolchildren remained mAAb-positive for biochemical autoantibodies (Supporting information, Fig. S1c). There was no difference between the prevalence of mAAb in Lithuanian ($n = 9$) and English schoolchildren ($n = 9$). However, within autoantibody positives, Lithuanian schoolchildren had three or four autoantibodies less frequently compared with English schoolchildren (3.4 versus 17%, $P = 0.043$, Fig. 3). Logistic regression analysis, including age and sex, confirmed the results obtained by all univariate analyses (data not shown).

Discussion

After new islet autoantibody measurement and analysis, Lithuanian schoolchildren have a lower frequency of PTPA and IA-2 β A compared with English schoolchildren. The number of Lithuanian schoolchildren with three or four islet autoantibodies was also lower. The frequency of ZnT8A and autoantibodies recognizing GAD (96–585) was similar in the two groups.

A large number ($n = 5913$) of schoolchildren were screened; autoantibody-positive schoolchildren were rare,

but some differences in the prevalence of antibodies were observed. In contrast to our previous paper, autoantibodies were measured concurrently for all schoolchildren. Assay variation cannot, therefore, explain the observed differences.

The intracellular region of IA-2 contains the PTP region. It is therefore expected that almost all individuals with antibodies that bind PTP would also bind the IA-2ic probe used for the harmonized assay. This was not the case for our study, which could be due to false positive results; for this reason, these samples were retested. Four of six were confirmed PTPA-positive, and these results were included in the above analysis. We note, however, that if IA-2A-negative schoolchildren are considered PTPA-negative there is no difference between Lithuanian and English schoolchildren for PTPA ($n = 3$ versus $n = 8$, respectively, $P = 0.135$). The thresholds used to assign antibody positivity for IA-2-related antibodies have been previously published applied to newly diagnosed children [8]; nevertheless, they were set using different populations of healthy individuals. When tested on the same population the harmonized threshold appears slightly more stringent than for PTPA and IA-2 β A. However, even if the threshold was lowered from 1.4 to 0.9 DK units, only three additional (one Lithuanian and two English) schoolchildren would be positive (data not shown). Although large numbers of samples have been tested from both populations, this still represents a small number of children with measurable antibody responses, limiting the power of the current study. The data from the previous paper ($n = 7$ statistical inferences) and current paper ($n = 13$ statistical inferences), IA-2A/PTPA/IA- β A tests and individual versus antibody numbers are not truly independent, therefore we have not applied correction for multiple testing. However, if Bonferroni correction was applied, the difference between IA-2 β A prevalence in English and Lithuanian schoolchildren would be replicated ($p_{corr} = 0.014$).

Samples for genetic analysis were not available. Genetics is also unlikely to explain the decreased prevalence of autoantibodies related to IA-2A, as human leucocyte antigen (HLA) class II haplotypes associated with type 1 diabetes were similar in Lithuanian schoolchildren compared with other European Caucasian populations [10]. However, it should be noted that higher-risk HLA haplotypes were more frequent in people with diabetes in Sweden compared to Lithuania [11]. Although ZnT8A were not measured in all individuals, previous unpublished data suggest that among non-diabetic children single ZnT8A-positive individuals were rare. In the current study, we were unable to assess the prevalence of schoolchildren single autoantibody-positive for ZnT8A or autoantibodies to GAD (96–585) due to this testing strategy.

We were also unable to confirm current progression to clinical disease for these schoolchildren. However, studies of the offspring of people with diabetes show that young children with multiple autoantibodies develop diabetes during childhood [12], suggesting that most of these children will progress to diabetes. Recent data suggest that risk of diabetes is equally high in young children with multiple autoantibodies from the general population [13]. Our study found a similar prevalence of multiple islet autoantibodies to this study from Bavaria (0.3%), despite a difference in the age of the children tested.

We previously speculated that the incidence of type 1 diabetes would rise rapidly in Lithuania due to similar frequencies of multiple islet autoimmunity in the school-child populations [2], and indeed incidence in Lithuania doubled between 1989–93 and 2004–08, whereas in England it rose by only a third [1]. However, the incidence of diabetes in England is still twice as high as in Lithuania [1], suggesting that the subtle differences in the autoantibody profiles between the populations may be important, particularly in determining progression rate. The incidence of autoimmune diabetes in adults, however, is not well characterized in either population; Lithuanian schoolchildren with islet autoimmunity may be diagnosed as adults.

This study found no difference in IA-2A prevalence between Lithuanian and English schoolchildren in contrast to our previous findings, and this may be accounted for by subsequent optimization of assays. The harmonized assay currently employed for IA-2A appears to be more specific than the previous assay format. However, in the 2009 Diabetes Antibody Standardization workshop our harmonized and local assays for IA-2A performed similarly (specificity 97 *versus* 97, sensitivity 62 *versus* 64). In addition, at the time of the original report IA-2A-positive individuals were retested for IA-2A using the same method; after reanalysis there was still a difference between the cohorts, with seven of seven Lithuanian and 30 of 70 English schoolchildren defined as positive.

Both PTPA and IA-2 β A arise later in the humoral response [14] before type 1 diabetes and are associated with rapid progression [5]. Autoantibody-positive Lithuanian schoolchildren were positive for fewer autoantibodies than autoantibody-positive English schoolchildren. This suggests that English schoolchildren at risk of diabetes have experienced more epitope- and antigen-spreading than Lithuanian schoolchildren of a similar age, which could be due to differing environmental determinants. In addition, Swedish children at diagnosis with diabetes were positive for more autoantibodies than Lithuanian children, which supports differing pathogenesis in the two countries [11]. The overlap of ICA

and autoantibodies recognizing GAD (96A) suggests that ICA may be more effective at recognizing this higher diabetes risk epitope, and could explain the added benefit of ICA testing within TrialNet [3].

Broadly, these new results support our previous observation that certain aspects of the autoantibody profile associated with more rapid progression are different in Lithuanian compared with English schoolchildren. The optimum age and combination of islet autoantibodies to use for screening different populations for diabetes risk may vary, therefore; for example, GADA alone may be a good primary screen for Lithuanian schoolchildren.

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Disclosures

The authors declare that there are no conflicts of interest associated with this manuscript.

Author contributions

A. E. L. and C. C. acquired, collated and analysed the data. A. E. L. wrote the manuscript. D. M. and A. J. K. W. collated the original data set. A. J. K. W. devised this study. All authors supported data interpretation, reviewed and edited the manuscript and contributed to the discussion. A. E. L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Historic islet autoantibody testing (Marčiulionytė *et al.* 2001 [1]) is indicated for GADA, IA-2A, and IAA (circles with solid borders). Current islet autoantibodies testing for ZnT8A, GADA (96-585), IA-2A DK, PTPA, and IA-2βA (broken borders). ^a indicates a samples that could not be tested for at least one of the autoantibodies. Loss of multiple autoantibody positive status is indicated by red text. ^b indicates one individual from this category was considered multiple autoantibody positive (mAab +ve) with previous testing but is not with new method. mAab +ve individuals are indicated in bold blue text. a) addition of ZnT8A in previously Aab+ve individuals, b) addition of GAD (96-585) testing in previously GADA positive, c) addition of harmonized IA-2A testing in previously IA-2A positive, d) breaking down the IA-2A epitope responses.

Fig. S2. Addition of ICA to biochemical autoantibody testing is indicated, i.e. 2/3 indicates that 2 out of 3 individuals in this area of the diagram were ICA positive. Historic islet autoantibody testing (Marčiulionytė *et al.* 2001 [1]) is indicated for GADA, IA-2A, and IAA (circles with solid borders). Current islet autoantibodies testing for ZnT8A, GADA (96-585), IA-2A DK, PTPA, and IA-2βA (broken borders). ^a indicates a samples that could not be tested for at least one of the autoantibodies. Loss of multiple autoantibody positive status is indicated by red text. ^b indicates one individual from this category was considered multiple autoantibody positive (mAab +ve) with previous testing but is not with new methods. Individuals with more than 1 positive test are indicated in bold blue text. a) Historic GADA, IA-2A, and IAA testing with addition of ZnT8A b) Historic IAA testing with addition of ZnT8A, and new testing for GADA (96-585) and IA-2A (IA-2A DK, PTPA, and/or IA-2βA).